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Following high-dose chemotherapy for the treatment of malignant disease, reinfusion of peripheral blood stem cells collected during treatment with growth factors usually results in delayed platelet engraftment as compared to neutrophil recovery. The overall objective of this research proposal is to improve the capacity of stem cells to engraft the megakaryocytic lineage. To achieve this goal we carried out *ex vivo* expansion of megakaryocyte (MK) progenitors from human bone marrow (BM) and peripheral blood stem cells (PBSC). Thanks to the availability of the recently cloned thrombopoietin (TPO), a growth factor specific for the MK lineage, we obtained highly enriched MK cultures in both hematopoietic sources. A synergistic effect between TPO and low doses of interleukin-3 (IL-3), but not stem cell factor (SCF), was observed in bone marrow cultures. Neither IL-3 nor SCF, at the concentrations used, enhanced TPO-stimulated megakaryocytopoiesis in PBSC. These observations will be used for *ex vivo* expansion of MKs to treat post-transplant-associated thrombocytopenia.

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INTRODUCTION

Following high-dose chemotherapy for the treatment of malignant disease, reinfusion of peripheral blood stem cells collected during treatment with growth factors usually results in delayed platelet engraftment as compared to neutrophil recovery (1-4). The overall objective of this research proposal is to improve the capacity of stem cells to engraft the megakaryocytic lineage. To achieve this goal mobilized peripheral blood stem cells (MPBSC) from forty patients randomized to receive either rGM-CSF or the new recombinant growth factor PIXY321 will be assessed for their capacity to undergo megakaryocytopoiesis. At the time the proposal was submitted, in view of the unavailability of a growth factor specific for the megakaryocyte (MK) lineage, we proposed to use serum from aplastic patients (AS) to stimulate megakaryocytopoiesis in the presence or absence of pleiotropic cytokines. In view of the variability in the megakaryocytic activity of the AS, we proposed to compare the results obtained with MPBSC to those obtained with twenty normal bone marrows (BM) and twenty fetal cord blood (FC) samples. Our main goal was to determine the best methodology of *ex vivo* expansion of the megakaryocytic lineage for purposes of cell transplant.

After over thirty years of unsuccessful attempts by several researchers to purify a growth factor specific for the MK lineage, five groups reported the successful cloning or purification of thrombopoietin (TPO). TPO was found effective at all levels of murine MK proliferation, nuclear and cytoplasmic maturation and platelet production (5-9). This growth factor was deemed of considerable importance for the treatment of thrombocytopenias following irradiation and chemotherapy. We were fortunate to secure the availability of TPO from Zymogenetics, for research purposes, and have substituted this cytokine for the AS proposed in the original submitted project.

BODY OF PROGRESS REPORT

Materials and Methods:

Preparation of low density non-adherent mononuclear cells (MNCs): Bone marrow (BM), fetal cord blood (FCB) and MPBSC samples were collected in accordance with the guidelines of the Institutional Review Board on Human Subjects. BM, obtained from the femur of hematologically normal patients having total hip arthroplasty, was collected in a special anticoagulant mixture designed to prevent platelet activation and containing final concentrations of 50 U/ml preservative-free heparin, 1 mM Na_2EDTA, 1 mM adenosine, 2 mM theophylline, 2.2 μM prostaglandin E_1 and 0.1 mg/ml DNase I. Marrow cells were repeatedly extracted from bone fragments with a modified MK medium (10) which consists of Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (Dulbecco's PBS, Gibco) containing 13.6 mM Na citrate, 11 mM dextrose, 1 mM theophylline, 1 % bovine serum albumin, 2.2 μM PGE_1 and 0.1 mg/ml DNase I. Following homogenization by passage through a 21 gauge needle, low density cells were extracted with the use of Ficoll-Paque as described (11). Cells resuspended in MK medium were centrifuged at 380xg through a 10 % human serum albumin cushion in PBS to reduce platelet contamination. Residual red cells were lyzed with NH_4Cl as described (12) and the remaining cells recovered by

centrifugation through a 10 % human serum albumin cushion. Adherent cells were discarded following overnight incubation in α-thioglycerol-free Iscove Modified Dulbecco's medium (IMDM) containing 10 % fetal bovine serum (FBS). All culture media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and incubation carried out at 37°C in a 5 % CO_2 fully humidified atmosphere.

FCB, collected in the special anticoagulant was centrifuged at 200xg for 10 min and the platelet-rich plasma discarded. Buffy coat and red cells were then submitted to Ficoll separation and the remaining steps carried out as for bone marrow cells. Frozen MPBSC were rapidly thawed at 37°C and this was followed by the dropwise addition of thawing medium consisting of IMDM, 25% FBS, 12.5 U/ml heparin and 1.25 mg% DNase I. Residual red cells were lyzed, and remaining MNCs were washed and treated as described above for the elimination of adherent cells.

Purification of CD34+ cells: CD34+ cells were purified by positive selection using the CD34 magnetic cell sorting Mini-MACS kit (Myltenyi Biotec, Auburn, CA) in accordance with the manufacturer's recommendation. A recovery of about 60 % CD34+ cells was obtained with a purity of 86.4 % ± 1.5 S.E.M (n=6) on the basis of flow cytometric analysis following staining with PE-anti-CD34 (HPCA-2).

Culture conditions: Low density non-adherent mononuclear cells (MNC) and purified CD34+ cells were cultured for 12-14 days at 37°C at concentrations of 10^6 and $5x10^4$ cells/ml, respectively, in an IMDM-based medium with 1 % human serum albumin and 2.5 % normal human serum which was found to be necessary for MK cultures. The concentration of MNCs and purified CD34+ cells seeded contained equivalent concentrations of CD34+ cells. In order to prevent the inhibitory effects on MK growth of transforming growth factor-ß, ß-thromboglobulin and platelet factor 4 released from activated platelets (13-15), normal serum was obtained by recalcification of citrated platelet-free plasma. Before TPO was made available to us, cultures were supplemented with 10 % AS. TPO (Zymogenetics Corp.) was then used at a concentration of 50 U/ml (10 U = TPO quantity which stimulates one-half maximal proliferation of BaF3/mpl cells) which yielded the maximal concentration of MKs. Concentrations of IL-3 and Stem Cell Factor (SCF) (R&D Systems, Minneapolis, MN), were 0.16 ng/ml (0.4-1.6 units based on 1 unit=ED50 of TF1 cell line proliferation) and 50 ng/ml, respectively.

Cell labeling and flow cytometric analysis: The relative frequency of MK progenitors was determined at day 1 on MNC and CD34+ cells. Cell aliquots were treated with 200 pkat chymopapain (Knoll Pharm. Co., Lincolnshire, IL) This treatment detaches most platelets and platelet fragments from cells which otherwise would stain with the anti-CD41a antibody (anti-GPIIb/IIIa). After washing the cells were double-stained with phycoerythrin (PE) conjugated-anti-CD34 (HPCA-2, Becton-Dickinson) and fluorescein isothiocyanate (FITC) conjugated anti-CD41a (Immunotech-Amac, Westbrook, ME) and analyzed by flow cytometry. Negative controls were PE-anti-mouse IgG_1 and FITC-anti-mouse IgG_1 used at equivalent IgG_1 concentrations. The relative frequency of mature MKs was determined following 12-14 days culture by flow cytometric analysis of cells stained with FITC-anti-CD41a.

Flow cytometric analysis was performed using a Coulter ELITE dual laser flow cytometer. Fluorescence attributable to FITC- and PE-labeled antibodies was determined using excitation by an argon laser operating at 488 nm and adjusted to 0.3 W. Emission from fluorescein and PE was measured using band pass filters of 530 ± 15 nm and 575 ± 15 nm, respectively. The per cent positive cells was calculated by substracting the percent positive of the isotype control within the same integration region.

Statistical analysis: The mean number of CD41a+ cells per seeded CD34+ cells and the mean ploidy index were compared across study conditions using two factor analysis of variance (ANOVA) with sample and study condition as the two factors. If the ANOVA p-value was less than 0.05, then pairwise comparisons were done between each pair of groups using the paired t-test with statistical significance indicated when $p < 0.05$. Separate analyses were done for mononuclear cells and for CD34+ cells. Stroma-free and stroma non-contact conditions were compared using the paired t-test.

Results and Discussion:

Cultures of marrow and fetal cord blood in the presence of aplastic serum (AS): The relative frequency of CD41a+ cells, expressing the $\alpha_{IIb}\beta_3$ integrin, of BM and FCB cultures, corresponded to that of cells labeled for vWF, which is a selective marker for MKs. The CD41a+ cells were also negative for CD14, specific for monocytes. Microscopic examination did not reveal the presence of vWF+ platelets adhering to cells. These data confirm that the CD41a+ cells are MKs.

Our results did not show a correlation between the percentage of CD34+ cells at day 1 and the percentage of CD41a+ cells (MKs) at day 12 for either marrow or FCB (Table I). Despite the low percentage of CD34+ cells in FCB (0.95 % ± 0.13 SEM) as compared to BM (3.47 % ± 0.49 SEM) (Table 1), a 10-fold higher percentage of CD41a+ cells was obtained in FCB (19.96 % ± 3.48 SEM) than in BM (2.03 % ± 0.46 SEM). When normalized to an equal amount of seeded mononuclear cells, the absolute number of CD41a+ cells obtained in FCB was 5-fold larger than in BM. When the absolute number of CD41a+ cells obtained at day 12 was calculated in function of a given amount of CD34+ cells, the results, considered as CD34+ "MK activity", unequivocally showed that CD34+ cells yielded 20-fold more MKs in FCB then BM cultures (Table I). The ploidy distribution for MKs from eight BM cultures was 2N, 63 ± 5 % SEM; 4N, 16 ± 2 % SEM; 8N, 14 ± 2.5 % SEM; 16N, 6 ± 1.3 % SEM; 32N, ≤ 1 %. The ploidy distribution for FCB MKs was not statistically different from that of BM MKs.

Table I. MONONUCLEAR CELL CULTURE: YIELD OF CD41a+ CELLS FROM BM AND FCB

Samples	% CD34+, day 1*		% CD41a+ day 12*		N** CD41a+ at day 12 per 10^7 seeded MNC		N** CD41a+ at day 12 per 10^5 seeded CD34+	
	BM	FCB	BM	FCB	BM	FCB	BM	FCB
1	1.51	1.02	0.83	5.42	$0.76x10^5$	$7.11x10^5$	$5.07x10^4$	$69.7x10^4$
2	4.11	0.84	2.26	18.70	$1.81x10^5$	$10.38x10^5$	$4.3x10^4$	$122.6x10^4$
3	3.18	0.49	2.64	33.77	$2.15x10^5$	$5.85x10^5$	$6.76x10^4$	$119.4x10^4$
4	3.28	1.63	3.2	11.60	$2.88x10^5$	$16.86x10^5$	$8.78x10^4$	$103.4x10^4$
5	2.90	0.72	1.26	32.89	$0.73x10^5$	$8.41x10^5$	$2.53x10^4$	$116.8x10^4$
6	4.77	1.37	1.24	22.30	$1.1x10^5$	$6.09x10^5$	$2.3x10^4$	$44.5x10^4$
7	2.23	0.63	0.56	14.42	$0.4x10^5$	$6.05x10^5$	$1.8x10^4$	$96.1x10^4$
8	5.8	0.92	4.26	20.65	$4.63x10^5$	$22.08x10^5$	$7.98x10^4$	$240.0x10^4$
Mean	3.47	0.95	2.03	19.96	$1.81x10^5$	$10.2x10^5$	$4.95x10^4$	$105.18x10^4$
± SEM	0.49	0.13	0.46	3.48	$0.5x10^5$	$2.06x10^5$	$0.95x10^4$	$23.89x10^4$
t-test	p=0.0012		p=0.012		p=0.014		p=0.036	

* Correlation between % CD34+ and CD41a+ for BM and FCB was r=0.688 and r=0.499, respectively.
** N, absolute number.

Culture of bone marrow MNCs and purified CD34+ cells in the presence of TPO, IL-3 or SCF.

In three different experiments no MKs were obtained when MNCs were cultured in serum-free media in the presence of TPO alone or TPO in combination with IL3 or SCF. Addition of serum or heparinized platelet poor plasma to TPO-treated MNCs cultures resulted in significant MK production. Therefore, all the experiments were carried out in the presence of 2.5% normal serum and 50 U/ml of TPO which were found optimal for MK growth.

IL-3 at low concentrations (0.16 ng/ml) potentiated the effect of TPO on MK growth from either MNCs or CD34+ cell cultures. The IL-3 effect was more pronounced with purified CD34+ cells than with MNCs since the increase ratio of MKs was 3 and 1.4 fold respectively (Fig. 1). The absolute numbers of MKs obtained per seeded CD34+ cell in CD34+ cultures were 0.02 ± 0.002 SEM, 1.14 ± 0.21 SEM, and 3.86 ± 0.78 SEM in the presence of IL-3, TPO, and IL-3+TPO, respectively (n=4). These results indicate synergism between IL-3 and TPO. The relative frequency of MKs was 32.1 ± 3 SEM and

55.8 ± 3 SEM (n=11) in TPO-stimulated cultures of MNCs and CD34+ cells, respectively, and was not affected by IL-3. The synergistic effect of IL-3 with TPO is therefore due to a general cell proliferation. In two separate experiments, MNCs were cultured in the presence of TPO and increasing concentrations of IL-3. The results showed that 1.6 ng/ml was the minimal IL-3 concentration which induced maximal MK proliferation. The numbers of MKs obtained per seeded CD34+cells were 4, 11.5 and 10 when cultures were performed using 0.16, 1.6 and 16 ng/ml respectively.

The addition of SCF to TPO did not have any effect on MK growth in cultures of either MNCs or purified CD34+ cells (Fig. 1). While IL-3 potentiated the effect of TPO in CD34+ cells when added in combination with SCF, IL-3 did not enhance TPO activity when added with TPO in MNCs (Fig. 1).

In all conditions, proliferation of MKs was significantly higher in MNCs than in purified CD34+cells (Fig. 1).

Preliminary results with FCB MNC and purified CD34+ cells yielded similar results.

Culture of MPBSC in the presence of TPO, IL-3 and SCF: The generation of MKs from MPBSCs harvested from 16 female breast cancer patients treated with either PIXY321 (Immunex) or GM-CSF was compared. Patients underwent leukapheresis after four days of cytokine therapy and again during cytokine-stimulated recovery from myelosuppressive doses (4gm/m^2) of cyclophosphamide (CPA). Non-adherent mononuclear cells (MNC) were cultured with 50 U/ml thrombopoietin (TPO, Zymogenetics) alone, or with 0.16 ng/ml IL-3 and/or 50 ng/ml stem cell factor (SCF). The percent CD34+ cells at day 1 and CD41a+ (MK) cells at day 12 were measured by flow cytometry.

There was no significant difference seen in the MK/10^3MNC yields between PIXY or GM-CSF stimulated harvests. PBPCs collected during cytokine stimulated recovery from CPA had a higher %CD34+ ($p=0.009$) and a greater MK/10^3MNC yield ($p=0.02$) compared to PBPCs primed by cytokine alone. The MK/10^3MNC yield correlated with the %CD34+ ($r=0.775$, $p=1.3\times10^{-5}$) (Table II).

Table II. CULTURE OF MOBILIZED PERIPHERAL BLOOD STEM CELLS: EFFECT OF TPO, IL-3 AND SCF

	%CD34+	MK/10^3 MNC SEEDED
	Median (Range; n)	
PIXY321	0.1 (0.03-0.2; 9)	18 (2-32; 9)
CPA+PIXY321	0.4 (0.05-1.4; 8)	33 (5-200; 7)
FOLD INCREASE	4.0 (0.5-14; 8)	3.0 (1.3-7.8; 7)
GM-CSF	0.3 (0.04-0.9; 7)	19 (2-63; 7)
CPA+GM-CSF	1.1 (0.25-4.7; 7)	98 (23-1240; 5)
FOLD INCREASE	5.2 (1.2-27.5; 7)	5.2 (1.1-19.7; 5)

The addition of IL-3, SCF, or both did not significantly increase the MK yield. Thus, MKs can be generated in vitro from peripheral blood when cultured with TPO; MK/10^3MNC yields were directly related to the percentage of CD34+ cells seeded.

CONCLUSIONS

In our first experiments, we used aplastic sera (AS) to supplement our cultures in order to achieve optimal megakaryocytopoiesis. Under these conditions, FCB appeared more efficient than BM in generating MKs, on the basis of the absolute number of MKs obtained per seeded CD34+ cell.

As TPO became available, we used it in the presence and absence of IL-3 and SCF in cultures of BM MNCs and purified CD34+ cells. Only IL-3 exhibited a synergistic activity with TPO in cultures of both mononuclear cells (MNCs) and purified CD34+ cells. This enhancing effect of IL-3 on the absolute number of megakaryocytes (MKs) per seeded CD34+ cell is due to a general proliferation of all cell types since the relative frequency of MKs was not affected by IL-3. In all conditions, higher proliferation of MKs was obtained with cultures of MNCs than CD34+ cells. This effect of MNCs, probably due to growth factor-secreting accessory cells was also observed on MK ploidy in the presence of TPO and IL-3. Due to the presence of accessory cells, MNCs appear more efficient in producing MKs than purified CD34+ cells. Nonetheless, purified CD34+ cells should be considered for *ex vivo* expansion in clinical trials since malignant cells can be practically eliminated by CD34+ selection (16). The potentiating effect of IL-3 on MK progenitors is of particular interest in view of its effect in supporting a basal level of megakaryocytopoiesis in the absence of TPO (17).

The results obtained with MPBSC cultures from 16 patients show that MKs can be generated in vitro from peripheral blood when cultured with TPO; MK/10^3MNC yields were directly related to the % CD34+ cells seeded. SCF did not potentiate TPO activity, similarly to BM cultures. Unlike BM cultures, however, IL-3 did not significantly potentiate TPO activity. This may be due to the relatively low concentration of IL-3 used (0.16 ng/ml). In our future MPBSC cultures, we are planning to use higher concentrations of IL-3 (up to 16 ng/ml) and assess its activity on TPO-induced generation of MKs. The findings will help in designing an *ex vivo* expansion protocol of MKs for possible use in supplementing transplants of hematopoietic progenitors in cases of slow platelet engraftment.

The timely availability of TPO enabled us to avoid using potentially hazardous heterologous sera from aplastic patients to supplement our cultures. Since the TPO supplied by Zymogenetics is a purified material with stringent testing of its biological activity, we shall mainly concentrate our efforts in comparing its activity in two hematopoietic sources, bone marrow cells and MPBSC. The latter has indeed been proven the material of choice for transplanting breast cancer patients (1, 18-20). This change in our "Statement of Work" (page 22 of our proposal) was deemed necessary in view of the seminal discovery of thrombopoietin.

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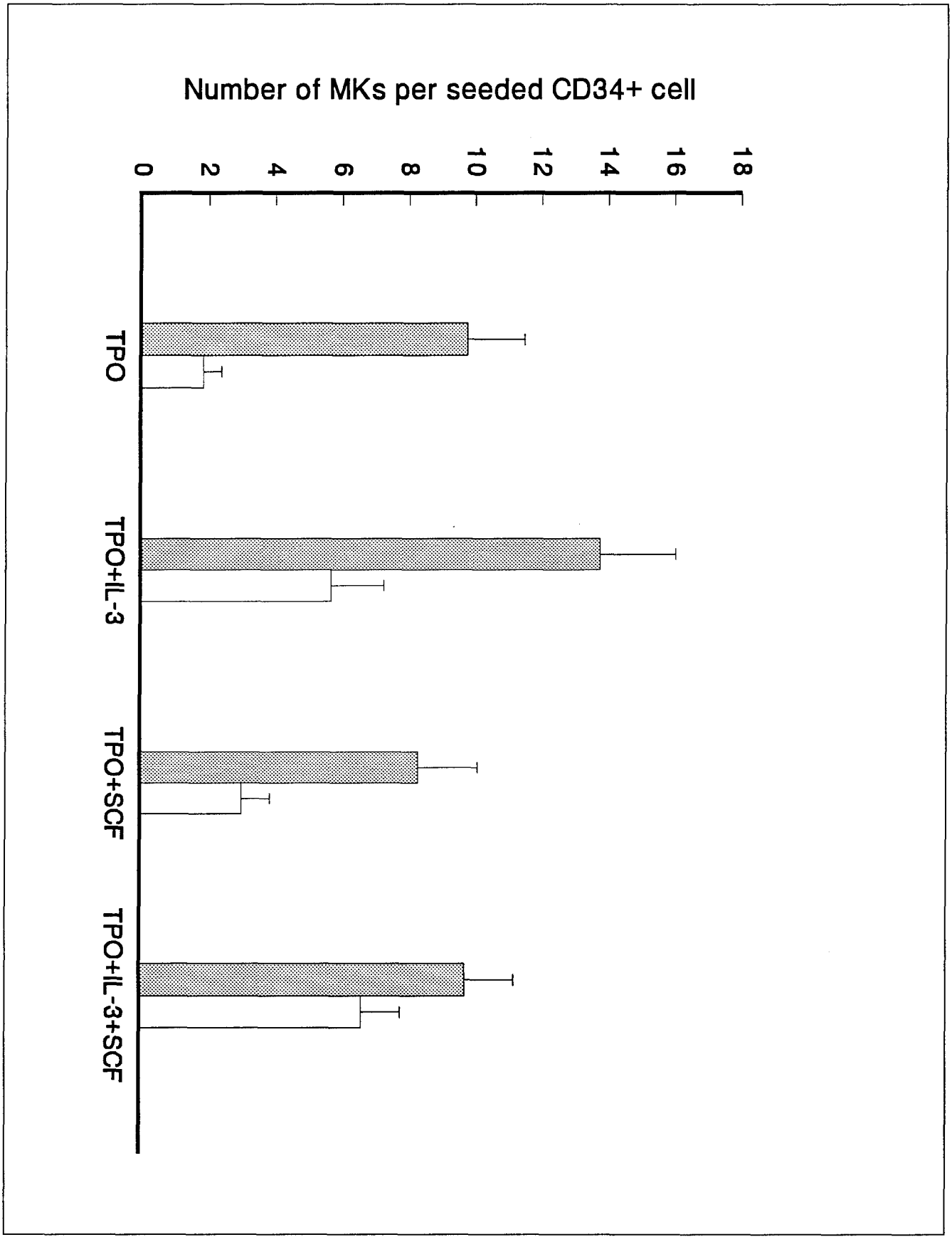
APPENDIX

Fig. 1. Effect of TPO, IL-3 and SCF on MK proliferation in cultures of MNC and CD34+ cells. *Shaded bars,* mononuclear cells; *Empty bars,* CD34+ cells. Mean (± SEM) number of CD41a+ cells per seeded CD34+ cell by study condition. For MNC cultures, analysis of variance (ANOVA) $p=0.003$. Pairwise comparisons: $p<0.05$ for TPO+IL-3 versus each of TPO, TPO+SCF and TPO+IL-3+SCF. For CD34+ cultures, ANOVA $p=0.03$. Pairwise comparisons: $p<0.05$ for TPO versus each of TPO+IL-3 and TPO+IL-3+SCF. For each of the four conditions, $p<0.05$ for MNC vs CD34+.


Number of MKs per seeded CD34+ cell
0
2
4
6
8
10
12
14
16
18
TPO
TPO+IL-3
TPO+SCF
TPO+IL-3+SCF